

Overexpression of focal adhesion kinase in vascular endothelial cells promotes angiogenesis in transgenic mice

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Abstract

Objective: Focal adhesion kinase is implicated in the regulation of cell adhesion, migration, survival, and cell-cycle progression. However, the functions of focal adhesion kinase in endothelial cell (EC) in vivo remain unclear. This study aims to examine the role of FAK in EC function and angiogenesis in vivo by transgenic mice approach.

Method: We generated transgenic mice which overexpressed chicken FAK in vascular endothelial cell under the control of the Tie-2 promoter and enhancer. FAK transgene was detected by RT-PCR, immunoprecipitation, and Western blot. The effect of FAK overexpression on angiogenesis was determined using skin wound healing and ischemia skeleton muscle models.

Results: Expression of FAK transgene was detected in all vessel-rich tissues. Expression of FAK protein was verified by antibody specific for the exogenous chicken FAK in lung homogenates and isolated EC. In the wound-induced angiogenesis model, the number of vessels in the granulation tissue of healing wound was significantly increased in the transgenic mouse compared to that of wild-type control mice. Similarly, in the ischemia skeleton muscle model, the density of capillaries was significantly increased in the transgenic mouse.

Conclusion: These results indicate that FAK may play an important role in the promotion of angiogenesis in vivo.

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Keywords: Focal adhesion kinase; Angiogenesis; Transgenic mouse; Endothelial cells

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1. Introduction

Angiogenesis is fundamental to embryonic development and organogenesis, as well as to the pathogenesis of many diseases including coronary heart disease, cancer and

inflammation [1]. Vascular endothelial cells (ECs) play a pivotal role in angiogenesis, functioning as transducers and effectors of local environmental signals for vessel formation [2]. EC sprouting is the initial step in angiogenesis and requires cell migration into the extracellular matrix (ECM) beneath the basement membrane [3]. Recent studies indicate that angiogenesis is regulated by signals derived from growth factor receptors and integrins [4,5]. Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that plays a key role in integrin-mediated signal transduction pathways [6–8]. Upon activation by integrin-mediated cell adhesion, FAK associates with a number of SH2 domain-containing signaling molecules, including Src family kinases [9–12], p85 subunit of

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phosphoinositide-3 kinase (PI3K) [13], phospholipase C- γ [14] and Grb7 [15]. Interactions of FAK with these signaling molecules trigger a number of downstream signaling pathways that regulate cellular functions including spreading, migration [16–19], survival [20–24] and cell cycle progression [18,25–27]. In addition to its well-established role in mediating integrin signaling, FAK may participate in growth factor receptor-induced signal transduction. Recent studies suggest that FAK associates with epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors through its N-terminal domain [28]. Thus, FAK may function as a point of convergence in signaling pathways triggered by integrins and growth factor receptors, both of which are important in regulating various angiogenesis-related cellular functions.

Several studies suggest that FAK is important for angiogenesis. FAK knockout mice die in utero at embryonic day 8.5 with extensive defects in generation (vasculogenesis) and subsequent growth (angiogenesis) of vasculature [29]. Increased EC migration into a wounded monolayer correlated with increased tyrosine phosphorylation and kinase activity of FAK [30]. Activation of vascular endothelial growth factor (VEGF) receptor-2 by VEGF-A induced the association of FAK with PI3K, which mediates porcine aortic ECs migration [31]. Angiopoietin-1, another stimulator of angiogenesis, also increased FAK phosphorylation during angiogenesis in vitro [32]. Interestingly, a recent report showed that the Src-dependent formation of a signaling complex of FAK and integrin $\alpha v \beta 5$ was essential for VEGF-stimulated angiogenesis in mice in vivo [33].

Although there is abundant in vitro data suggesting an important role for FAK in angiogenesis, little is known about the in vivo function of FAK in ECs during angiogenesis. To investigate the physiological role of FAK for vascular development and angiogenesis in vivo, we established transgenic mice that overexpress FAK specifically in vascular ECs, using the Tie2 promoter and enhancer. This was the first well-characterized, EC-specific promoter that drives target gene expression throughout embryogenesis and adulthood in ECs [34,35]. Our results show that overexpression of FAK in ECs can promote angiogenesis during wound healing and in response to ischemia.

2. Materials and methods

2.1. Generation and identification of transgenic mice

Transgenic mice were generated using pSPTg.T2FpAXK (pg52) plasmid (kindly provided by Dr. Thomas Sato, University of Texas Southwestern Medical Center), which contains the Tie2 promoter and enhancer and a SV40 PolyA signal (personal communication). A 3.2-kb full-length chicken FAK cDNA was excised with *PvuI* and *HincII* from pBS-FAK [36]. A *NotI* linker was ligated to *HincII* site

of this fragment and the resulting product was then subcloned into pg52 at the *NotI* site. The Tie2-FAK transgene was then excised with *SalI* from the vector backbone for injection. Gel-purified Tie2-FAK transgene was microinjected into pronuclei of fertilized mouse eggs from C57BL/6 X CBA/J mice, which were then transferred into pseudopregnant female mice. All study protocols were approved by the Institutional Animal Care and Use Committee of Cornell University and conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Potential founder mouse strains and their offspring were identified by PCR and Southern blot analysis with genomic DNA prepared from tail biopsies. Routine genotyping was performed by PCR with the use of a forward primer corresponding to the Tie2 promoter (5'-GGGAAGTCGCAAAGTTGTGAGTT-3') and a reverse primer corresponding to the FAK cDNA (5'-CTCCATGCCGGATAATGC-3') to amplify a 300-bp fragment spanning the junction between the Tie2 promoter and FAK cDNA. The PCR conditions were as follows: 1 cycle at 94 °C for 10 min, 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, 1 cycle at 72 °C for 10 min. Southern blot analysis was performed with *HindIII*-digested mouse tail genomic DNA using the PCR product as a probe.

2.2. Reverse transcription–polymerase chain reaction (RT–PCR) analysis

Total RNA was isolated from mouse lung, heart, liver, spleen, kidney and brain with TRIZOL (Invitrogen, CA) and purified with RNAeasy (Qiagen, CA) kit according to the manufacturer's instructions. The isolated total RNA was digested with DNase at room temperature for 10 min to remove potential DNA contamination. The samples were then reverse transcribed to cDNA, amplified by PCR, and resolved on an agarose gel. The PCR primers and conditions were the same as described above for genotyping. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reverse transcription control (primers: 5'-ACGACCCTTCATTGACCTC-3' and 5'-CTTTCAGAGGGGC-CATCCAC-3').

2.3. Isolation of ECs from transgenic mice

Primary ECs were isolated by positive selection using immunobead precipitation from the lungs of 6–12-week-old mice as previously described [37]. Briefly, lungs were harvested, minced, then digested with Type I collagenase (2 mg/ml, Worthington Biomedical, Lakewood, NJ) at 37 °C for 45 min. The digested tissue was mechanically dissociated using vigorous flushing through a metal cannula, passed through a 70- μ m filter (Becton Dickinson Labware, Becton, MA), then centrifuged at 400 \times g for 8 min at 4 °C. The cells were re-suspended in cold Dulbecco's phosphate-

buffered solution (DPBS), then incubated with rat anti-murine CD31 (PECAM-1, clone MEC 13.3, BD Biosciences, San Jose, CA)-coated magnetic beads (M-450, sheep anti-rat IgG Dynabeads, Dynal, Great Neck, NY) at 15 μ l/ml for 10 min at room temperature. The beads were washed several times in cold isolation medium (high glucose Dulbecco's modified Eagle's medium containing 20% fetal bovine serum, penicillin, streptomycin (at standard concentrations) and 0.02 M HEPES), re-suspended in growth medium (isolation medium supplemented with heparin (100 μ g/ml), endothelial cell mitogen (100 μ g/ml, Biomedical Technologies, Stoughton, MA), L-glutamine, non-essential amino acids and sodium pyruvate at standard concentrations), and cultured in 0.1% gelatin (Sigma)-coated 100 mm tissue culture dishes at 37 °C in 5% CO₂. When the cells reached 80–90% confluence, they were detached using trypsin–ethylenediaminetetraacetic acid (EDTA). The cells were then centrifuged in isolation medium, re-suspended in DPBS then incubated with rat anti-murine CD102 (ICAM-2, BD Biosciences)-coated Dynabeads for 10 min at room temperature. After washing in isolation medium, the beads were re-suspended in growth medium and cultured in 100 mm culture dishes at 37 °C in 5% CO₂. Confluent cells were used at passages 1–3.

2.4. Flow cytometry

Confluent cultures were detached using trypsin–EDTA, centrifuged and the cell pellet was re-suspended in PBS containing 1% bovine serum albumin (BSA). The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated rat anti-murine CD102 and phycoerythrin (PE)-conjugated rat anti-murine CD31, with appropriate isotype controls (BD Biosciences), on ice for 30 min. After washing twice in PBS/1% BSA, the cells were fixed in 1% paraformaldehyde in PBS. Analysis was performed using a FACScaliber (BD Biosciences). Cells were gated on forward and side scatter characteristics (linear scales). After applying appropriate compensation settings using isotype controls, 10,000 gated cells were counted and analyzed for positive fluorescence (log scale). The percentage of cells positive for FITC-CD102 and PE-CD31 was determined from dotplots, using the quadrant tool on the FACScaliber.

2.5. Immunoprecipitation and Western blot

The rabbit anti-FAK serum (α -KC) that recognizes FAK from different species has been described previously [9]. A new rabbit antiserum specific for chicken FAK (anti-cFAK) was prepared by Proteintech (Chicago, IL) using a synthetic peptide corresponding to chicken FAK amino acid 781–796 (see Fig. 3A). Anti-cFAK antibody was conjugated to protein A beads according to the manufacturer's instructions. Briefly, 0.5 ml antiserum was mixed with 2 ml protein A beads in 6 ml PBS and incubated at 4 °C overnight. Protein A beads were then transferred to a column and

washed with PBS and borate buffer (0.2 M sodium borate, pH 9.0). The protein A beads were then incubated with 20 mM dimethyl pimelimidate (Pierce Biotechnology, IL) at room temperature for 1 h. They were then washed two times with 0.2 M ethanolamine (pH 8.0) and PBS.

For most experiments, subconfluent cells or tissues isolated from mice were washed three times with ice-cold PBS and then lysed with modified RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 1% Triton X-100, 0.5% SDS, 0.1% EDTA, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 1 mM PMSF). Lysates were cleared by centrifugation and total protein concentrations were determined using the Bio-Rad Protein Assay (Hercules, CA). Immunoprecipitation was carried out by incubating lysates with anti-cFAK conjugated-protein A beads at 4 °C for overnight. The beads were then washed twice using RIPA buffer and once with PBS. After washing, the beads were boiled with loading buffer and resolved by SDS–PAGE. Western blotting was performed using α -KC (1:1000) as the primary antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000) as the secondary antibody. A chemoluminescent substrate (Amersham ECL) was used to detect the signal.

In some experiments, lysates from isolated ECs were analyzed directly by antibodies against FAK (C-20, Santa Cruz), Y397 phosphorylated FAK (Upstate) or vinculin (Sigma) at 1:1000 dilutions.

2.6. Wound healing assay

Mice were anesthetized with sodium pentobarbital (160 mg/kg) intraperitoneally for the surgical procedure, which was performed under aseptic conditions. Four full-thickness wounds were made on the backs of 12-week-old mice using a 2-mm skin punch. Seven days later, mice were euthanized and wounds were excised and fixed in 10% buffered formalin and stored for histology and immunohistochemistry (see below).

2.7. Hindlimb ischemia model

To create unilateral hindlimb ischemia, the femoral artery of 6-month-old mice was exposed by incising the skin overlying the medial femor, as described previously [38]. The femoral artery and vein were ligated proximally and all side-branches were dissected free. The overlying skin was then closed using surgical silk. Four weeks later, mice were euthanized and three biopsies of ischemic tissue were saved and fixed in 10% (w/v) buffered formaldehyde for histology and immunohistochemistry examination (see below).

2.8. Histology and immunohistochemistry

After euthanasia, complete postmortem examination was performed and tissue samples were collected. Tissue

samples were immediately fixed in 10% (w/v) buffered formaldehyde for overnight. Representative samples were then progressively dehydrated in ethanol, cleared with xylene and embedded in paraffin wax according to standard laboratory procedures. Sections of 4 μ m were stained with hematoxylin and eosin (HE) for routine histopathological examination with light microscopy. Paraffin-embedded 4- μ m sections of biopsied tissues were deparaffinized with xylene, rehydrated in 100%, 95% and 75% ethanol baths, and then treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. After blocking with 10% goat serum, samples were incubated with affinity purified polyclonal rabbit anti-CD31 (1:200) as primary antibody. After washing in PBS, the samples were incubated with biotinylated goat anti-rabbit IgG followed by streptavidin–HRP (ImmunoCruz Staining System, Santa Cruz, CA), using DAB as a substrate. The number of capillaries was counted in 10 random high (40 \times) magnification fields by a double-blinded veterinary pathologist.

2.9. Statistical analyses

Data are presented as mean \pm standard deviation. Means were compared by analysis of variance between groups (ANOVA). $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Generation of Tie2-FAK transgenic mice

To investigate the potential function of FAK in angiogenesis *in vivo*, we created transgenic mice that express FAK in vascular ECs under the control of the Tie2 promoter and enhancer. Tie2 is a vascular EC-specific receptor tyrosine kinase that plays an important role in the regulation of vascular network formation and remodeling. The Tie2 promoter and enhancer cassette has been well characterized and used for specific expression of exogenous genes in EC of the entire vasculature *in vivo* [34,35]. The cDNA encoding full-length chicken FAK was subcloned into the vector between the Tie2 promoter and enhancer (Fig. 1A), and used for generation of transgenic mice as described in the Materials and Methods. Two stable independent transgenic mouse lines (TG-19 and TG-34) were obtained and used for experiments.

The Tie2-FAK transgene was detected by PCR using oligonucleotides corresponding to the Tie2 promoter and FAK, as forward and reverse primers, respectively. As expected, this combination of primers amplified a band of 300 bp from the transgenic mice, but not the endogenous Tie2 or FAK gene of the transgenic or wild type (WT) mouse (Fig. 1B). We also confirmed the presence of the

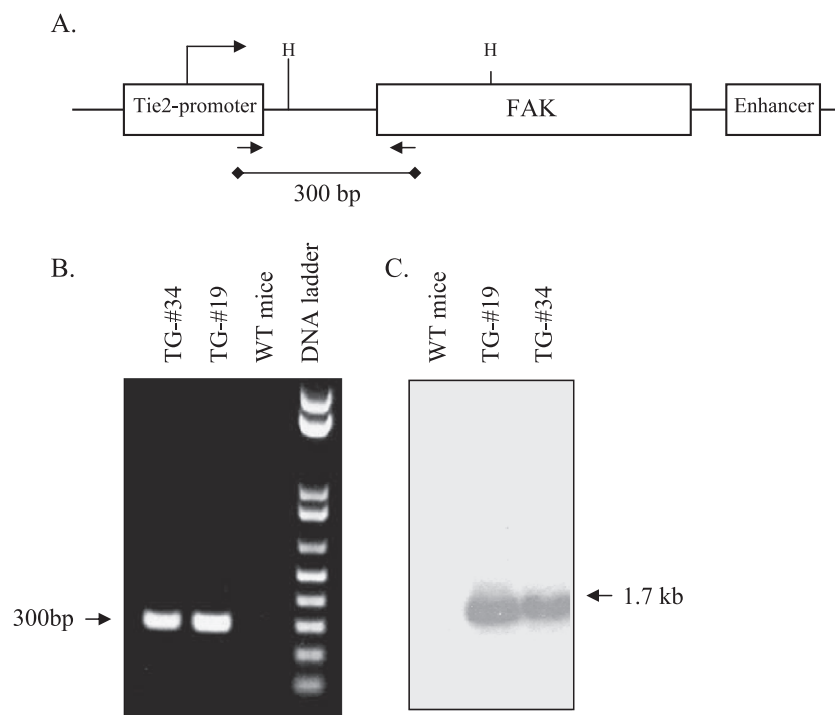


Fig. 1. Generation of Tie2-FAK transgenic mice. (A) Schematic representation of the FAK construct used to generate transgenic mice under control of the TIE2 promoter and enhancer cassette. The location of the primers (arrows) used in the PCR and the expected 300-bp PCR product and the restriction sites *Hind*III (H) used in digestion for Southern blotting are shown below and above, respectively. (B) DNA from tails of transgenic and WT mice were amplified by PCR and resolved on a 1% agarose gel, with DNA molecular weight markers, as indicated. The expected 300-bp band for FAK transgene is marked by an arrow on the left. (C) DNA from tails of transgenic and WT mice were digested by *Hind*III and then subjected to Southern blotting analysis using the 300 bp PCR product as a probe. The expected 1.7-kb band for FAK transgene is marked by an arrow on the right.

transgene by Southern blotting analysis using the 300-bp PCR product as a probe (Fig. 1C). The Tie2-FAK transgenic mice were indistinguishable from WT mice and they did not display any detectable developmental abnormalities. No macroscopic or microscopic lesions were found that could be attributable to the presence of the transgene. Normal mendelian inheritance of the transgene was observed when hemizygous mice were intercrossed. Together, these results suggested that overexpression of FAK in vascular ECs does not affect mice development.

3.2. Analysis of FAK transgene expression in the ECs

To evaluate expression of the transgene, we first examined mRNA expression levels of the transgene by RT–PCR using total RNA isolated from mouse lungs which are highly vascularized and contain abundant ECs. Oligonucleotides corresponding to the Tie2 promoter and FAK were used as the primer pair specific for the transgene, but will not amplify endogenous Tie2 or FAK mRNA (see Fig. 1B). We detected the FAK transgene RNA from transgenic, but not from WT mice. RNA of GAPDH (450 bp), a housekeeping gene, was detected in both RNA samples (Fig. 2A). Furthermore, bands were not detected when the reverse transcription step was eliminated, indicating that the signals were not due to DNA contamination of the RNA samples (Fig. 2A, odd number lanes). We next examined transgene expression in other tissues with abundant ECs, using similar methods. The FAK transgene RNA was expressed in all tissues examined, including the heart, liver, spleen, kidney and brain (Fig. 2B). These results are consistent with a previous report showing that the Tie2

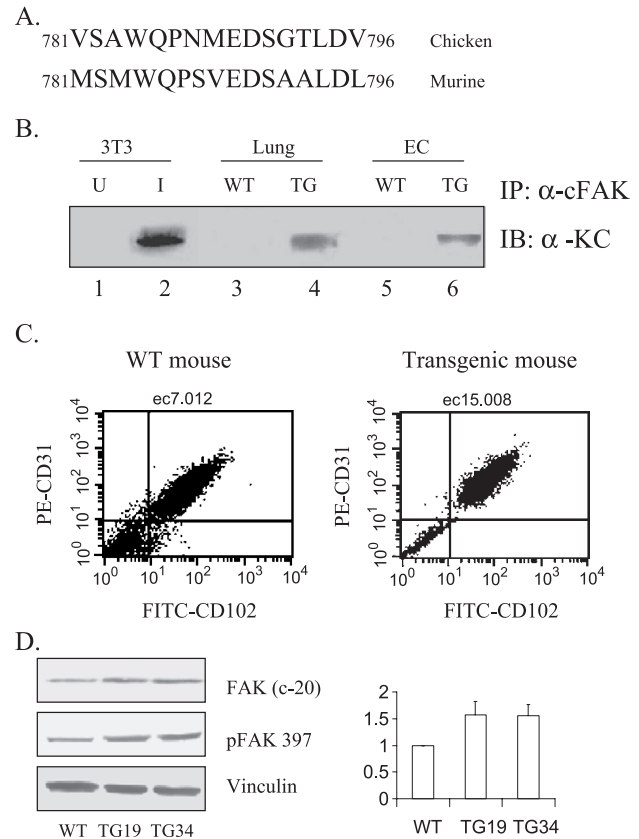


Fig. 3. Expression of transgenic FAK in vascular ECs. (A) Alignment of chicken FAK sequence with the corresponding sequence of murine FAK. (B) Cell lysates were prepared from FAK cells under non-induced and induced conditions, lungs of WT and transgenic mice, and isolated ECs of WT and transgenic mice, as indicated. Lysates were immunoprecipitated by anti-cFAK antiserum and blotted for FAK with α -KC. (C) FACS analysis of lung ECs from WT and transgenic mice, as indicated. Greater than 80% of cells are ECs, as shown by double positive staining for CD31-PE and CD102-FITC (upper right quadrant). (D) Lysates from lung ECs were analyzed by Western blotting using antibodies against FAK (top panel), PY397 of FAK (middle panel), or vinculin (bottom panel), as indicated. The relative expression of FAK (normalized to vinculin expression and then to FAK in WT mice) were obtained from three independent experiments (average and S.D.) and shown on the right.

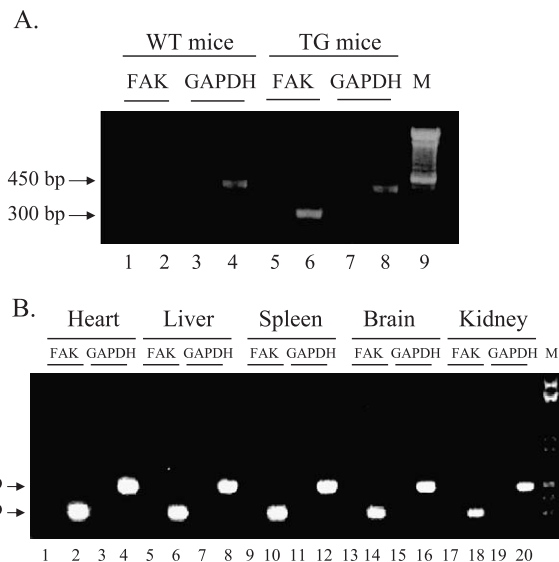


Fig. 2. Detection of FAK transgene by RT–PCR. Total RNA was isolated from lungs of transgenic and WT mice (A) or various tissues of transgenic mice (B), as indicated. RNA samples were analyzed with (even number lanes) or without (odd number lanes) the reverse transcription step prior to PCR. The expected 300-bp band for FAK transgene and the 450-bp band for GAPDH control are marked by arrows on the left.

promoter and enhancer directs gene expression in ECs of the entire vasculature [34,35].

FAK is highly conserved among the species. We tested various available FAK antibodies, but none could distinguish chicken and mouse FAK. Therefore, to demonstrate expression of the exogenous chicken FAK protein in the transgenic mice, we prepared an antiserum using a synthetic peptide corresponding to residues 781–796 in chicken FAK. This is the most divergent region from the mouse FAK sequence (Fig. 3A). The specificity of this antiserum (anti-cFAK) was tested using NIH3T3 cell clone with tetracycline-inducible expression of exogenous chicken FAK (designated FAK cells) [26]. The anti-cFAK specifically immunoprecipitated chicken FAK from FAK cells under induced conditions but not the endogenous mouse FAK in the uninduced cells, as shown by Western blotting analysis

using α -KC, which reacts with FAK from all species (Fig. 3B; lanes 1 and 2). We then examined exogenous chicken FAK expression in the lungs from the transgenic and WT mice. The exogenous chicken FAK was detected in the anti-cFAK immunoprecipitates of lysates from transgenic mice, but not from WT mice (lanes 3 and 4), confirming that the transgene is expressed at the protein level.

To confirm that the FAK transgene was expressed in ECs, we also isolated ECs from the lungs of transgenic and WT mice using magnetic bead immunoisolation, as described in the Materials and Methods. After two rounds of positive selection with beads coated with anti-murine CD31 and anti-murine CD102, we routinely obtained a yield of 80% ECs, as determined by the percentage of cells double positive for CD31 and CD102 by flow cytometry (Fig. 3C). The identity of ECs was further verified by their cobblestone morphology and immunofluorescent staining with anti-VE-cadherin (data not shown). Immunoprecipitation assays using the purified ECs confirmed exogenous FAK expression in the transgenic mice (Fig. 3B, lanes 5 and 6). Analysis of the lysates by an antibody that recognizes both transfected chicken FAK and endogenous mouse FAK shows an approximately 50% increase in the total FAK

expression for both transgenic strains, suggesting that the transgene is expressed at about 50% level as the endogenous mouse FAK (Fig. 3D). Furthermore, total FAK that are phosphorylated at Y397 is also increased by about 50%, suggesting that the chicken FAK transgene was regulated in a similar manner as the endogenous mouse FAK. Together, these results demonstrate expression of FAK in ECs of transgenic mice under the control of the Tie2 promoter.

3.3. Enhancement of wound-induced angiogenesis by FAK overexpression

Previous in vitro studies have suggested potential roles for FAK in EC migration and angiogenesis [30,39]. To investigate this possibility in vivo, we examined wound-induced angiogenesis in the transgenic mice and WT mice. The rate of wound healing of back incision wounds was similar between the transgenic and WT mice. Histological examination by staining with H/E did not show any apparent differences in the healing wounds. To quantify angiogenesis in the granulation tissue, vascular density was evaluated by CD31 staining of ECs 7 days after wounding. CD31 staining was increased in granulation tissue of

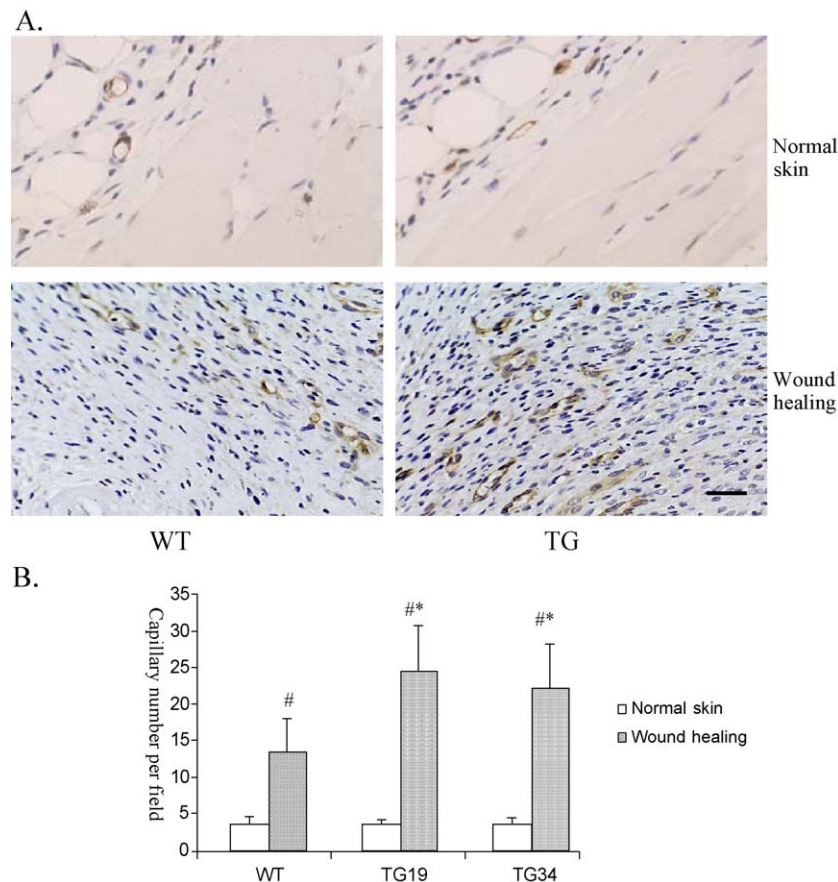


Fig. 4. Overexpression of FAK increases capillary density in wound healing. (A) Four excision full-thickness wounds were made with a skin biopsy punch on the back of 3-month-old mice ($n=4$). One week later, the capillary density was measured with immunohistochemistry using polyclonal anti-CD31 antibody (bottom two panels). Sections of normal skin were also stained with anti-CD31 (top two panels) (bar=50 μ m). (B) Capillary density was quantified by counting the number of vessels per field for 10 fields and expressed as mean \pm S.D. vessel number per field (*vs. control, $p<0.05$; #vs. respective normal skins, $p<0.05$).

transgenic, compared with WT mice (Fig. 4A). The number of vessels per field were significantly ($P<0.05$) increased (by about 80%) in wound of transgenic, compared with WT mice (Fig. 4B). In normal skin, we did not observe any difference in the number of vessels between WT and transgenic mice. These results suggested that overexpression of FAK in the ECs of transgenic mice may promote angiogenesis in response to wounding.

3.4. FAK overexpression increase capillary density in hindlimb ischemia

We also examined the effects of FAK overexpression on angiogenesis using the hindlimb ischemia model [38]. The left femoral artery and vein were ligated to induce unilateral hindlimb ischemia in transgenic and WT mice. All mice survived after surgery and appeared healthy during the following 4 weeks. The ischemic femoral muscles were then harvested and examined for capillary density by immunohistochemistry, using CD31 as an EC marker to determine the extent of angiogenesis. Vessel

density was significantly increased in femoral muscles from transgenic compared to WT mice (Fig. 5A). Quantitative analysis revealed a statistically significant increase of about 120% ($P<0.05$) in the density of capillaries of transgenic mice compared to WT mice (Fig. 5B). In normal muscle, we did not observe any difference in the number of vessels between WT and transgenic mice. Together with the data from the wound-induced angiogenesis model, these results strongly suggest that FAK stimulates angiogenesis in vivo.

4. Discussion

Angiogenesis and neovascularization are complex processes controlled by signals derived from receptors for ECM proteins and growth factors. Consistent with its role in mediating signal transduction by integrins and growth factor receptors, recent studies have shown that FAK is important for EC migration and angiogenesis in vitro [32,39]. However, direct demonstration of FAK's role in

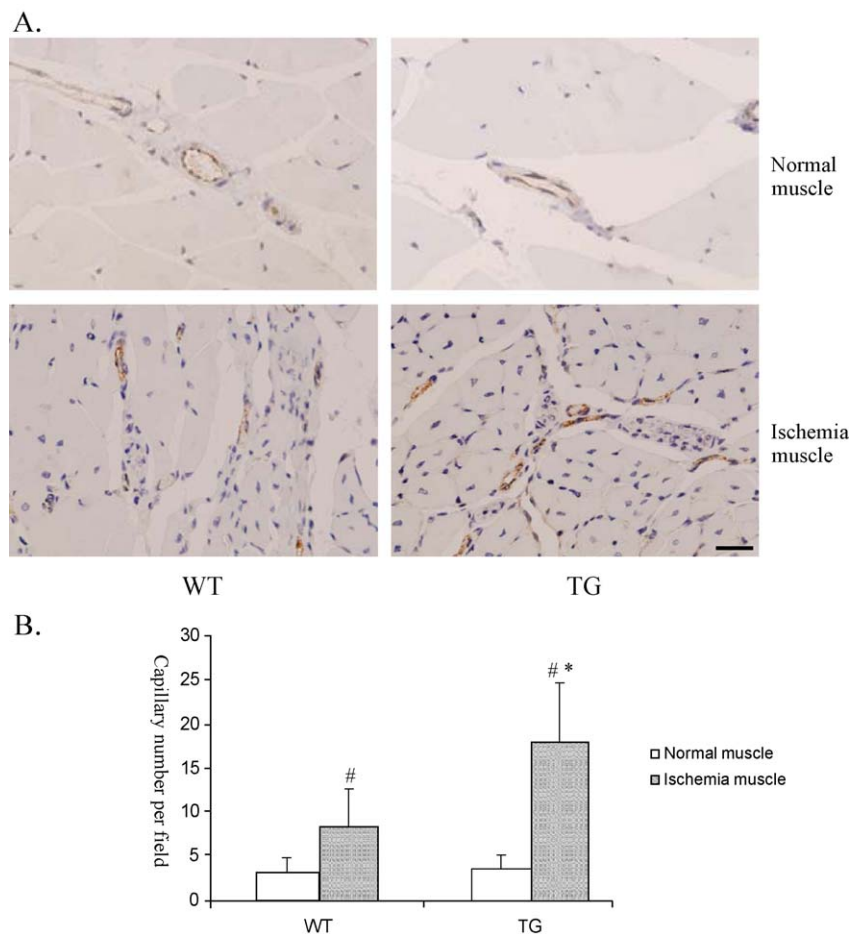


Fig. 5. Overexpression of FAK increases capillary density in ischemic hindlimb muscle. (A) The left femoral artery of 6-month-old transgenic ($n=9$) and WT ($n=6$) mice was ligated. Four weeks later, capillary density of the ischemic muscle biopsies was evaluated by immunohistochemistry using polyclonal anti-CD31 (bottom two panels). Sections of normal muscle were also stained with anti-CD31 (top two panels) (bar=50 μ m). (B) Capillary density was quantified by counting the number of vessels per field across at least 10 random fields and expressed as mean \pm S.D. vessel numbers per field (*vs. control, $p<0.05$; #vs. respective normal muscles, $p<0.05$).

angiogenesis remains to be established *in vivo*. In this study, we generated a new transgenic mice line that targeted overexpression of FAK to vascular ECs using the Tie2 promoter. We showed that the FAK transgene is expressed in all vessel-rich tissues examined and in isolated ECs from the lungs. Although they developed normally, transgenic mice exhibited increased angiogenesis in the wound healing and ischemic hindlimb angiogenesis assays. These results provide direct support for a role of FAK in angiogenesis *in vivo*.

Dynamic interactions between ECs and ECM are essential for angiogenesis [4]. This interaction is mediated mainly by integrins, which are composed of a large family of non-covalently linked α and β heterodimers [40,41]. Currently, 11 members of the family ($\alpha_{1-7}\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_6\beta_4$) are known to be expressed in ECs at various times, and most have been suggested to be involved in the regulation of angiogenesis [42]. For example, VEGF-induced angiogenesis was inhibited by antibodies against $\alpha_1\beta_1$ and $\alpha_2\beta_1$ [43]. β_1 -knockout mice die *in utero* at E5.5 before the vascular system forms, preventing assessment of its role in angiogenesis. However, the differentiation of ECs derived from β_1 -/- embryoid bodies is delayed [44,45]. In α_5 -knockout mice, the formation of extraembryonic and embryonic vessels was severely impaired [46]. These studies strongly suggest a potential role of FAK in angiogenesis because it is a major mediator of integrin signaling. FAK may also participate in growth factor receptor signaling. A recent study has shown that FAK is required for angiogenesis induced by VEGF and basic fibroblast growth factor (bFGF) *in vitro* [33]. Taken together with data shown here, FAK may function as one of converging points in the signaling pathway triggered by integrins and growth factor receptors, that are important in the regulation of angiogenesis.

Deletion of the FAK gene in mice results in embryonic lethality with major defects in embryonic vasculogenesis and angiogenesis [17,29]. Interestingly, overexpression of FAK in ECs did not affect these processes during embryonic development, although we cannot exclude subtle differences between transgenic and WT embryos. Overexpression of FAK may promote pathophysiological angiogenesis, as shown here in the wound healing and ischemic hindlimb models, despite minimized effect on embryonic vasculogenesis and angiogenesis under physiological conditions. It is interesting to note that hypoxia is a strong inducer of angiogenesis by stimulating VEGF [47]. Hypoxia occurs in wound healing and ischemia may affect VEGF levels, although we did not evaluate these here. Hypoxia-induced angiogenesis in these models may be further enhanced by overexpression of FAK in ECs, because FAK is important in VEGF-induced signaling [48–51].

Consistent with a lack of effect of overexpression of FAK on embryonic development, we also noted that the capillary numbers in normal skin and skeleton muscle are not changed in transgenic mice compared with their WT

littermates (Figs. 4 and 5). This indicates that FAK is not a limiting factor for blood vessel development under normal conditions. However, it may become a limiting factor to mitogenic and/or migratory responses in ECs under an increased angiogenesis conditions in skin wound healing and muscle ischemia. Thus overexpression of FAK could overcome the limitation and allow enhanced angiogenesis in transgenic mice. These considerations are based on that the transgene chicken FAK is regulated and functions in a similar manner as the endogenous mouse FAK. This is highly likely given the extremely conserved sequences of FAK among different species [52]. Indeed, we found that the ectopic chicken FAK was phosphorylated on PY397 as efficiently as the endogenous FAK, which caused a corresponding increase in the total Y397 phosphorylated FAK as the increase in total FAK protein (see Fig. 3D). Because autophosphorylation of FAK at Y397 is critical for both FAK regulation and function, these data suggest that the overexpressed chicken FAK behaved in a similar manner as the endogenous FAK.

The mechanisms by which FAK overexpression in ECs promotes angiogenesis in adult mice remain to be established. It is likely, however, that FAK's ability to promote EC migration contributes to it [18]. EC migration is one of the first steps of angiogenesis. Increased EC migration correlated with increased FAK phosphorylation and activity [30]. FAK signaling pathways also regulate cell survival and cell cycle progression [26,52,53]. These may also contribute to angiogenesis by FAK *in vivo*. Future studies will be necessary to clarify these potential mechanisms and to delineate FAK downstream pathways involved in its regulation of angiogenesis *in vivo*.

The availability of the FAK transgenic mice model described here will likely to be valuable tools in both basic and clinical researches. It will provide a useful model to further understanding the role of FAK and its downstream signaling pathways in angiogenesis *in vivo* by analysis of various FAK mutants in a similar manner. Furthermore, crossing the FAK transgenic mice with different integrin and/or FAK EC conditional KO mice in the future will facilitate the analysis of integrin-FAK signal transduction pathways in angiogenesis. Lastly, both anti- and pro-angiogenic therapy are currently one of the most active research areas for treatment of various diseases such as cancer and coronary heart disease. Elucidating the precise molecular mechanism of angiogenesis including the role of FAK *in vivo* will likely contribute to the development of potentially novel strategy in angiogenesis based therapies.

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